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PRINCIPAL INVESTIGATOR: **Thomas J. Gill, M.D.**

CONTRACTING ORGANIZATION: **Massachusetts General Hospital
Boston, MA 02114**

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14. ABSTRACT

The goal of our research is to introduce a novel means to regenerate the articular cartilage and restore normal function of the joint. A strategy that can generate durable hyaline articular cartilage, which will be predominantly type II collagen, and is capable of integrating with the surrounding cartilage matrix (without fissures) could improve the long-term outcome of joint surface repair. Key findings are 1) Several modifications of photochemically crosslinked gels including: a) Adding fibrin to the type I collagen solution and photochemically crosslinking the combination gel using Riboflavin as the photoinitiator and blue light (458 nm) increases the bulk modulus of the gels; b) Performing the photochemical crosslinking under hypoxic conditions may improve cell survival; c) Cells are uniformly distributed throughout the crosslinked gels and the cells begin producing pericellular matrix by 2 weeks in vitro in the gels; and c) High cell survival and matrix production was noted in the poly(ethylene) glycol thiol-ene gels. 2) Cell tracking was verified in vivo with Dil labeled cells encapsulated in gels placed in nude mice for up to 8 weeks. 3) Formation of new cartilage matrix was demonstrated in vivo in mice using photochemically crosslinked gels and swine articular chondrocytes placed into cartilage rings. 4) Bone marrow-derived mesenchymal stem cells from swine were isolated and differentiated into osteogenic, adipogenic, and chondrogenic lineages with commercial differentiation media containing growth factors inducing differentiation. 5) Chondrocytes encapsulated in photochemically crosslinked hydrogels can survive the crosslinking and implantation process as shown in swine.

15. SUBJECT TERMS

Cartilage; articular; collagen gel; poly(ethylene)glycol gel; photochemical crosslinking

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INTRODUCTION

Injuries to the cartilage surfaces of joint are particularly problematic because, unlike bone and other vascular tissues comprising the joint, cartilage is avascular and possesses limited capacity for repair and self-regeneration. Consequently, injury to cartilage in the articulating joints from trauma results in scar formation and possible arthritic changes that can lead to pain, stiffness, and loss of structure and function [1-3]. These joint injuries not only limit physical activity and mobility of those afflicted, but the inability to move freely can cause deep psychological scars and loss of independence when individuals have to depend on family and healthcare providers for constant assistance to perform daily life functions. The level of functional capability in the injured limb and ultimate quality of life depend on the successful outcome of joint surface regeneration performed as a secondary procedure weeks or even months after the initial injury. The return of function and the probability of return to active duty rely on successful restoration of the entire joint including the articular surface, and therefore, joint function. Lesions in the joint surface are commonly treated with microfracture [4], autologous cell implantation (ACI) [5], or osteoarticular autograft transfer system (OATS) [6]. To date, however, the outcomes of many restorative procedures are very unsatisfactory and an improved method for joint repair is a clear unmet need in military medicine. **The goal of our research is to introduce a novel means to regenerate the articular cartilage and restore normal function of the joint.** A strategy that can generate durable hyaline articular cartilage, which will be predominantly type II collagen, and is capable of integrating with the surrounding cartilage matrix (without fissures) could improve the long-term outcome of joint surface repair. *The scope of this research is to develop regenerative medicine approaches involving biocompatible hydrogel scaffolds seeded with autologous cells that provide three-dimensional environments favorable for promoting chondrogenesis for joint surface repair.*[7-9]

In the previous annual report covering the first 12 months of this 48 month project, we reported on generating two candidate photochemically crosslinked hydrogels for encapsulating chondrocytes or chondrocyte precursor cells. Photochemically crosslinked gels were made using collagen as a natural protein gel and poly(ethylene) glycol thiol-ene gels as a synthetic gel. The photochemically crosslinked collagen gels demonstrated increased resistance to collagenase digestion over uncrosslinked gels, but had little effect on changing the bulk modulus (stiffness) of the gels. Adding a secondary chemical crosslinking step with 33mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 6 mM N-hydroxysuccinimide (EDC/NHS) for 1 hour prevented collagenase digestion over 24 hours and increased the bulk modulus nearly four-fold. The report for year 1 showed results of in vivo cartilage formation using these collagen gels photochemically crosslinked with either riboflavin and blue light or Rose Bengal and green light. Despite satisfactory cartilage formation, these gels were biomechanically weak. Although the secondary chemical crosslinking step increased the stiffness of the gels, this step had a negative effect on cartilage formation by encapsulated cells. Testing in year one of the PEG thiol ene gels showed changes in the shear moduli were related to the weight percent of the gels. New modifications of both types of gels were studied in year 2 according to the original algorithm proposed in the grant application and shown in Figure 1.

This annual report covering the second 12 months of the project reviews our progress testing the physical and chemical properties of these photochemically crosslinked hydrogels and their ability to promote chondrogenesis. The tasks referenced below pertinent to Year 2 under Task 1 include subtasks 1.1.a, 1.1.b, 1.1.c, 1.2.a, and under Task 2 include subtask 2.1.a.

Algorithm for Testing *In Vivo*

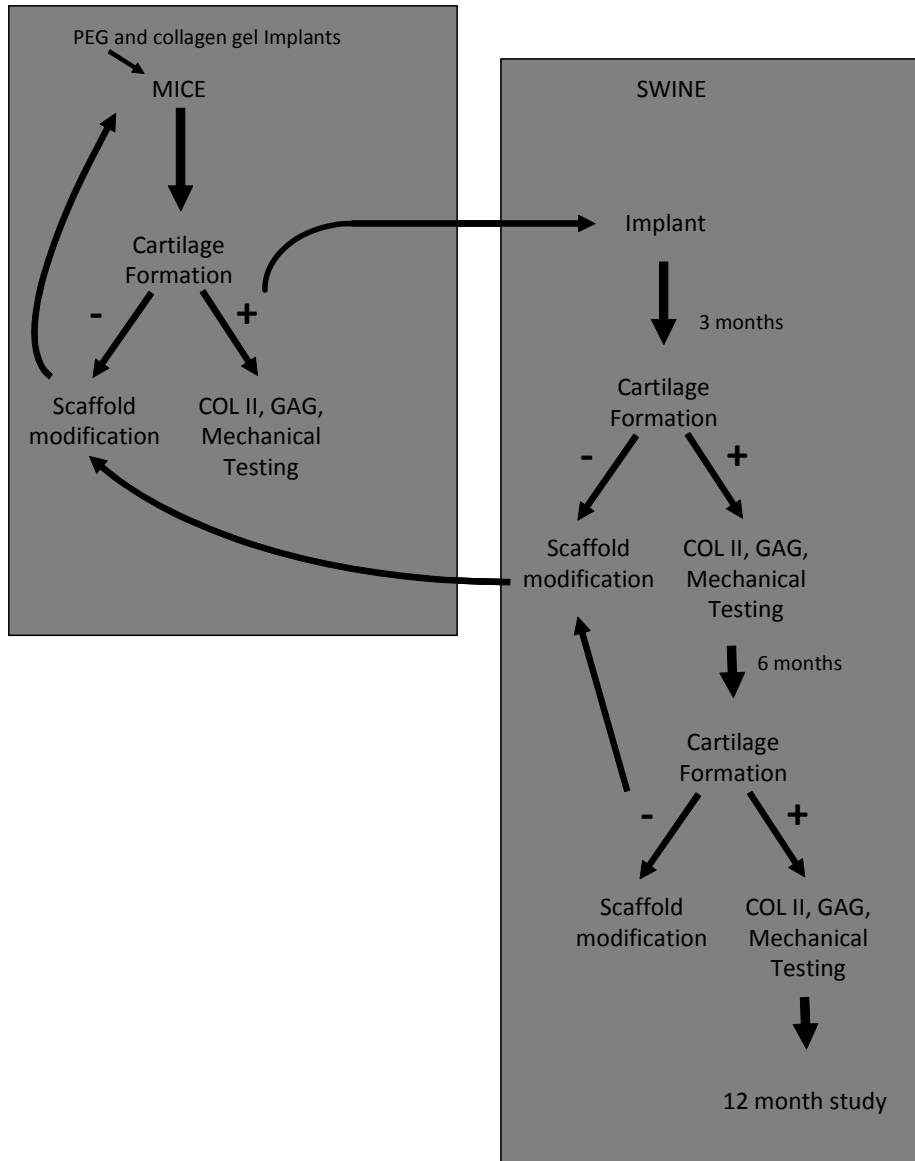


Figure 1. This is the testing paradigm proposed in the original grant application. The gels are made and tested in vitro and in vivo in mice (left box) prior to embarking on the large animal swine model (right box). If gels fail to perform in the mice they are not tested in swine. Additionally, if the gels should perform poorly in the early test phase in swine, the gels are reformulated and tested again in mice before re-embarking on the swine studies. Many changes outlined in this report describe changes to the gels so that large animals are not used unnecessarily for gels that have not been optimized.

BODY

Task 1 Test of photochemically crosslinked gels to produce cartilage and bone using chondrocytes and osteoblasts

Subtask 1.1.b Perform implantation of photochemically crosslinked collagen and PEG gels with chondrocytes and osteoblasts

- In vitro preparation and testing of photocrosslinked collagen gels for cytocompatibility and strength.

Result: The weak mechanical properties of gels made solely from rat tail type I collagen solution made us pursue other options to enhance the stiffness of the gels while, at the same time, permitting cartilage formation. Using the rat tail collagen we observed that some batches received from the vendor (BD Biosciences) did not gel, either spontaneously or when photocrosslinked. A change to bovine type I solution from Invitrogen was made resulting in more consistent gelation. A second strategy involved photochemically crosslinking fibrin gels. Bovine fibrinogen crosslinked with thrombin previously has been reported as a favorable scaffold for cartilage formation by encapsulated chondrocytes [10]. Studies over the past year included riboflavin (250 μ M) added to the fibrinogen/thrombin mixture and photochemical crosslinking with blue light (458 nm). A third formulation was prepared to take advantage of the properties of crosslinked collagen and fibrin by preparing a combination gel. Cell-seeded and unseeded gels were evaluated on a rheometer to determine the bulk moduli of the three types of protein hydrogels. The results are shown in Figure 2. Noncrosslinked fibrin (without cells) has a high bulk modulus (>2100 Pa) and photochemical crosslinking has little additional effect on the modulus. On the other hand, bovine type I collagen gel has a very low modulus and photochemical crosslinking has minimal effect on the gel modulus of the gel. Our previous results reported in year 1 show that the collagen gel is very cytocompatible and permits neocartilage formation. To take advantage of the properties of this characteristic and increase the modulus, we chose to add fibrin to the collagen gel. The addition of the fibrin to the collagen gel increases the modulus nearly 20 times over the collagen alone when the gel is photochemically crosslinked. The addition of cells to the gels drops the modulus of the gel somewhat, but the effect of the fibrin added to the collagen is still much higher than the collagen gel alone.

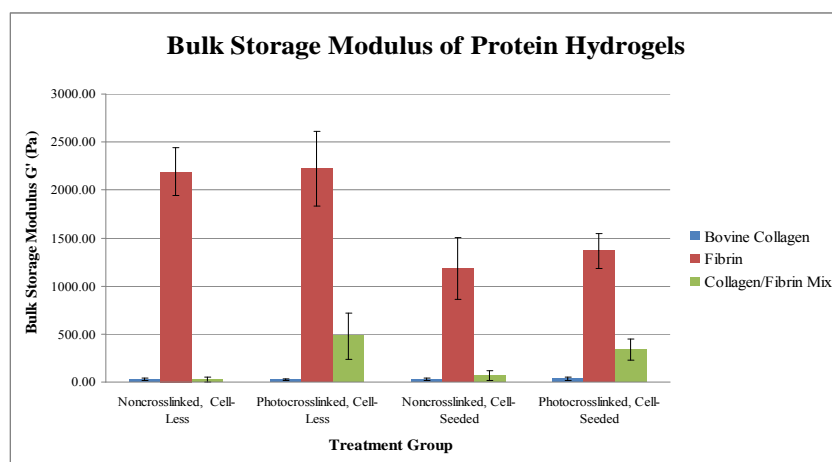


Figure 2. Bulk modulus (G') of 4% wt/vol bovine collagen gel, fibrin gel, and a combination collagen/fibrin gel. Gels were tested uncrosslinked and photochemically crosslinked using 250 μ M riboflavin and blue light. Gels without cells were tested and compared to gels containing cells (40 million/cc). Fibrin gel has the highest modulus and adding fibrin to the collagen gels increased the modulus of the collagen gel by nearly 20-fold.

Result: It is well known that the photochemical crosslinking process can cause the formation of reactive oxygen species (ROS) which can oxidize biomolecules and alter cell function. Although we have demonstrated above that cartilage can be formed when the crosslinking takes place in ambient air, we sought to minimize these negative effects by removing the oxygen present during the exposure to light. To test this, we performed a pilot experiment removing as much ambient oxygen as possible. This was accomplished by bubbling nitrogen gas through closed containers of the gel solutions and all reagents prior to composing the gels. Additionally, the gels were placed in a transparent vessel in which the oxygen was evacuated for 10 minutes with nitrogen gas. Gels were treated with and without light. Control gels were treated in ambient air with and without light exposure for comparison. The gels were placed in culture and evaluated 24 hours and 7 days after photocrosslinking. Live/dead assays were performed on the gels to assess the viability of the cells and histology was performed to evaluate the spatial distribution of the cells in the gels. The live/dead results showed that the cells survived the photochemical crosslinking process but the gels crosslinked under hypoxic conditions have fewer dead cells (Figure 3). Histological results showed that the cells were distributed uniformly throughout the gels. Furthermore, pericellular extracellular matrix was observed in the day 7 samples (Figure 4). From these data we concluded that the hypoxic conditions may permit higher cells survival during the photochemical crosslinking process. A full scale study is planned for the first quarter of year 3 to evaluate the fully formed cartilage matrix under these conditions.

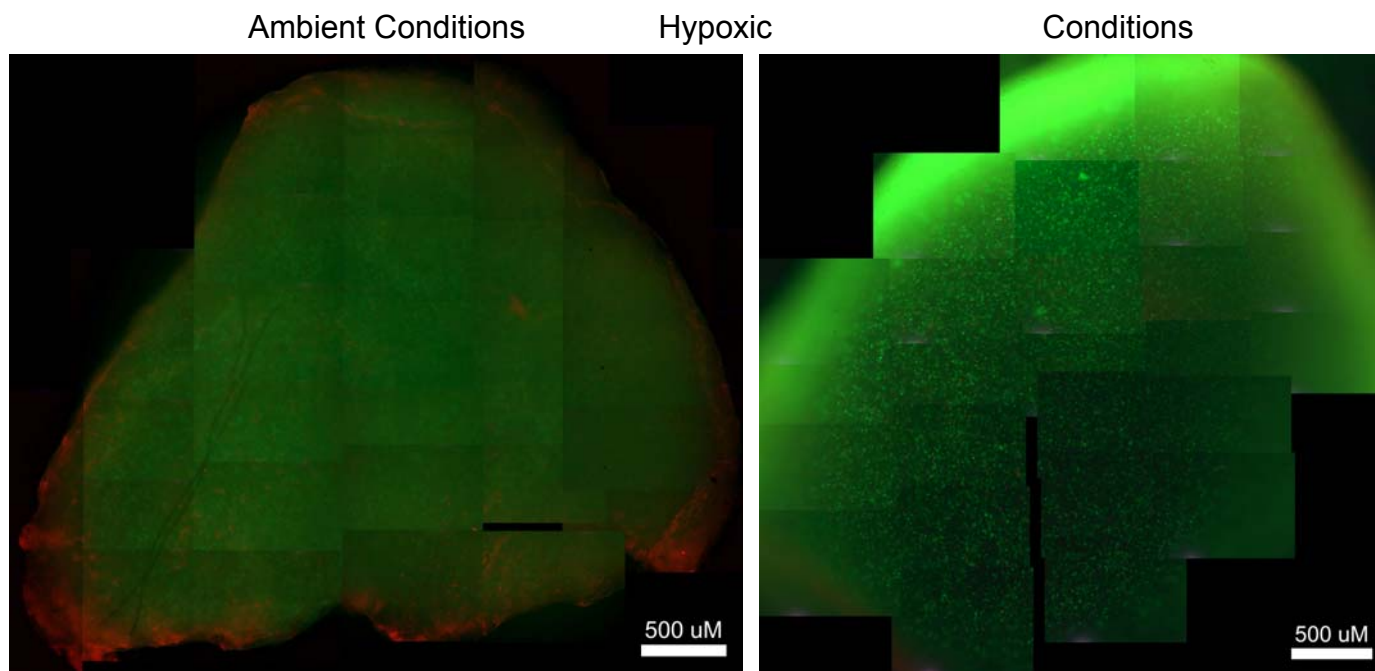


Figure 3. Swine chondrocytes encapsulated in fibrin gels photochemically crosslinked under ambient conditions (left) and hypoxic conditions (right) stained green (calcein AM) to indicate a live cells and red (ethidium homodimer-1) to indicate dead cells. This assay was performed 24 hours after photochemical crosslinking and the images demonstrate that the cells survive the encapsulation process (mostly green cells) under both conditions. In the specimen crosslinked under ambient conditions there are areas of dead cells around the periphery, and a small amount of dead cells in the interstices of the gel. There does not appear to be any dead cells in the gel crosslinked under hypoxic conditions.

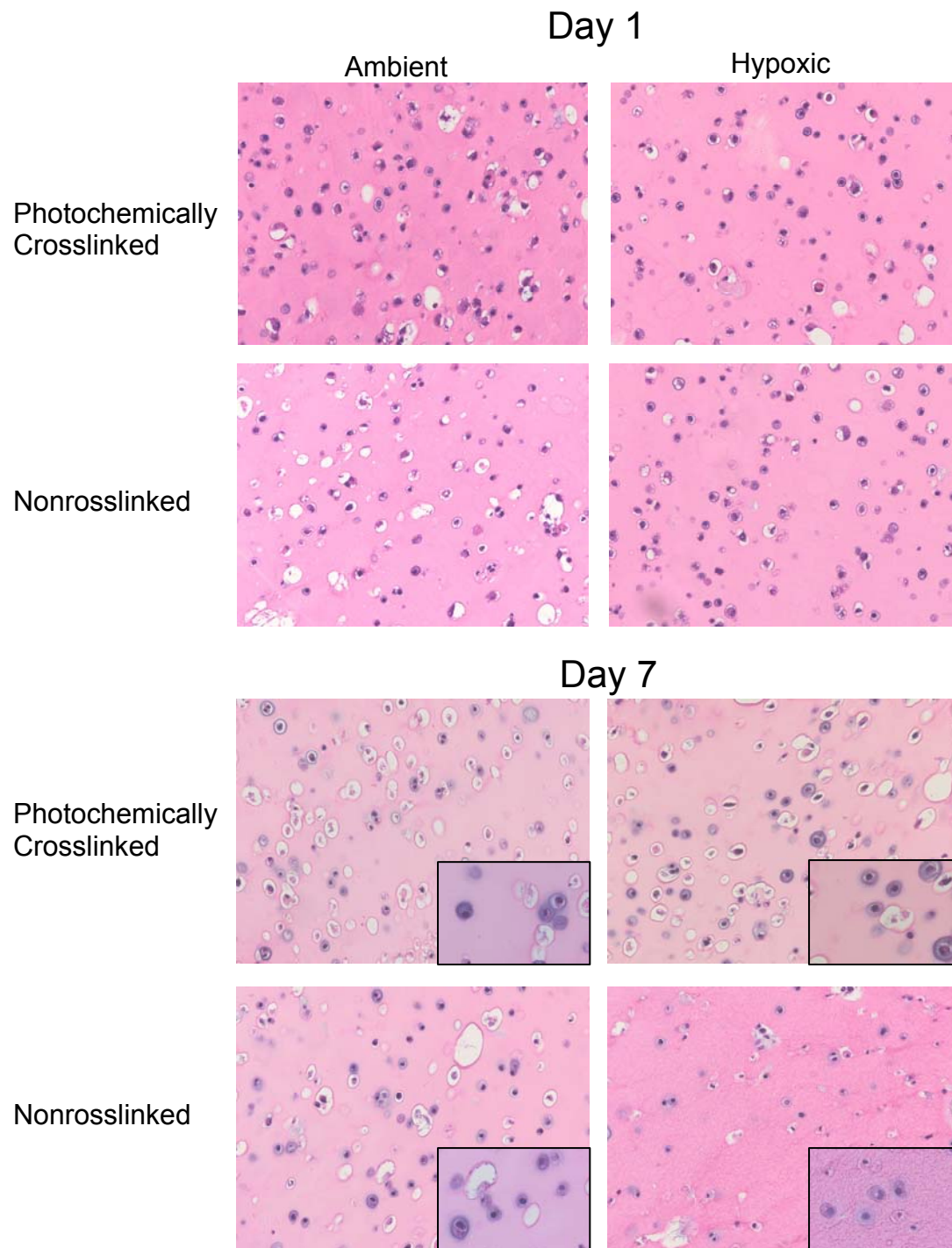


Figure 4. Histology of fibrin gels uncrosslinked and photochemically crosslinked with riboflavin and blue light under ambient air conditions (left column) and under hypoxic conditions (right column) at days 1 and 7 of in vitro culture (100x; H&E). The cells are distributed uniformly throughout both types of gels. The insets in the day 7 images (400x) show pericellular matrix formation as dark purple halos around the cells.

Result: Many studies involving implanted cells report an outcome, either positive or negative, without knowing if the cells survive or remain in the site with the intended function. For cartilage repair it is important to know whether or not the implanted cells remain in the site and make new extracellular matrix. This point is critical for cartilage repair where the cells can be washed away mechanically or unintentionally released from the scaffold. An experiment was performed where swine chondrocytes were labeled with the cell-tracking dye DiI, a red fluorescent cell membrane marker, for in vivo tracking. Cells were encapsulated in photochemically crosslinked gels placed in a cartilage ring simulating an articular cartilage defect. The rings were implanted in the subcutaneous space of nude mice for 1, 2, 4, and 8 weeks. At each time point rings were collected and imaged with a laser scanning confocal microscope to visualize whether or not the cells remained in the gel inside the cartilage ring. The images in Figure 5 show the results at 4 weeks from one control gel and two photocrosslinked gels. The implanted cells fluoresce red as shown in the Figures 5a, b, c, and the individual cells can be seen in figure 5d. Additionally, the cartilage ring did not contain labeled cells and the interface of the cell-seeded gel and the cartilage ring can be noted in Figures 5d and e.

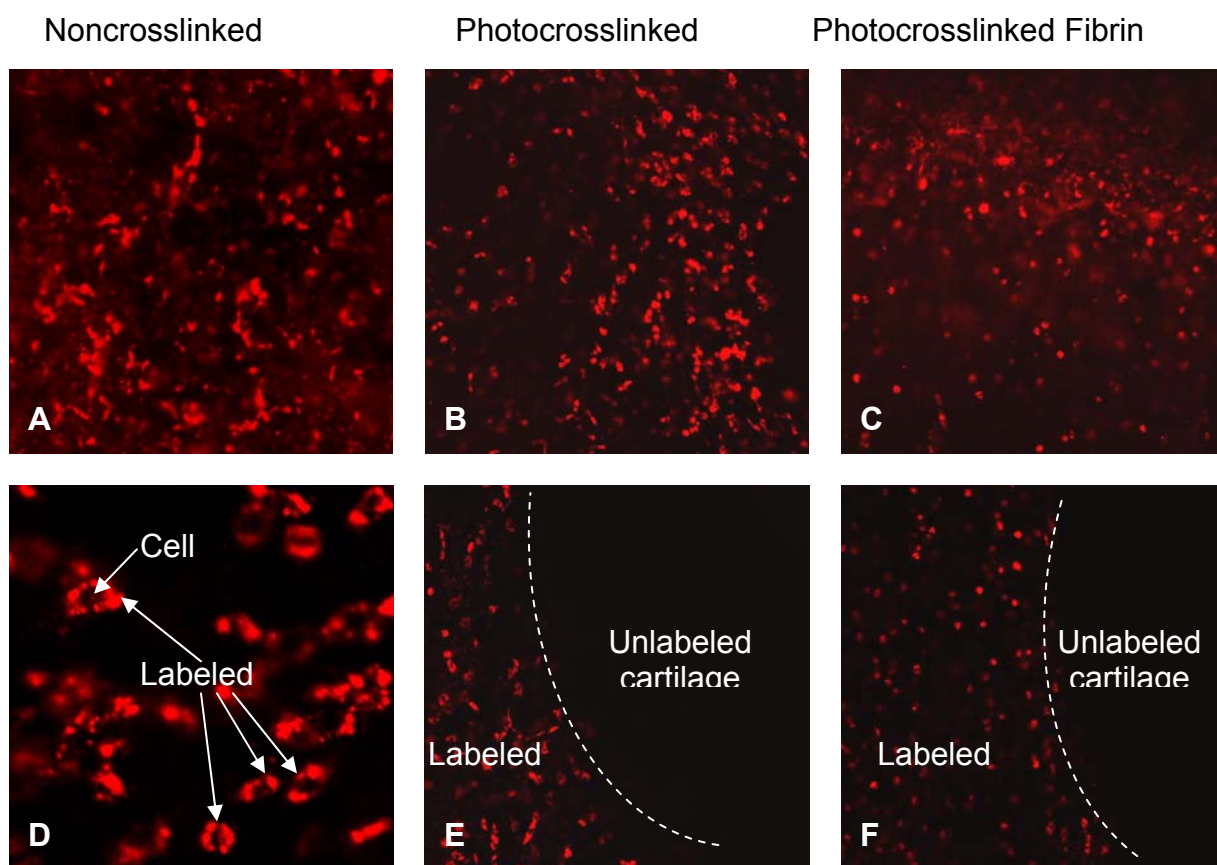


Figure 5: Laser scanning confocal images of chondrocytes encapsulated in noncrosslinked fibrin gel, photochemically crosslinked fibrin gel, and a photocrosslinked gel comprised of fibrin and collagen. These representative samples were imaged after 4 weeks of in vivo implantation showing the presence of cells that were labeled with DiI prior to implantation. Images a, b, and c demonstrated the distribution of the cells throughout the gels (20x magnification). Image d is a higher magnification showing individual cells and their nuclei. Images e and f show the labeled cells adjacent to the cartilage ring (interface marked with the white dotted line) in which the cells are not labeled (20x magnification).

- In vitro preparation and testing PEG gels for cytocompatibility and strength

Results: A poly(ethylene) glycol (PEG) hydrogel system has been developed to deliver cells to defect sites within the body. This system takes advantage of a radical-mediated chemical reaction that selectively bonds thiols to molecules containing carbon-carbon double bonds (“enes”). This thiol-ene reaction can be initiated with exposure to biologically compatible wavelengths of light ($\sim 365\text{nm}$, at $10\text{mW}/\text{cm}^2$) to form PEG hydrogel matrices that are crosslinked with short, enzymatically degradable peptides. Gelation of these synthetic 3D matrices can be accomplished in the presence of cells within minutes making it a suitable vehicle for cell delivery. Moreover, this chemistry leads to the formation of hydrogel niches whose structures can be tuned to promote chondrocyte survival and proliferation on a timescale commensurate with extracellular matrix (ECM) production for cartilage regeneration. As shown in Figure 6, chondrocytes exhibit high levels of survival when cultured in these thiol-ene systems up to 25 days. One tunable aspect of these hydrogels is the rate of enzymatic cleavage of the crosslinking peptides, which can be tailored by altering the amino acid sequence of the site. In our work with porcine chondrocytes, we have selected a previously used non-specific matrix metalloproteinase (MMP) sensitive sequence, GPQGIAGQ but this sequence can be easily altered to either increase its susceptibility to enzymatic cleavage and matrix degradation or to slow these processes. Figure 7 shows a histological image of chondrocytes producing ECM in a MMP degradable system. Another tunable aspect of this synthetic hydrogel system is the matrix density and stiffness. By using PEG monomers with different arm numbers and concentrations, it is possible to tune global physical properties of the resultant polymers. For example, we compared the calculated the Young’s modulus of hydrogels synthesized with PEG monomers containing different arm numbers. As shown in Figure 8, hydrogels made with 4-arm 5K PEG had moduli ranging from 6-100 kPa, whereas similar hydrogels made with 8-arm 10K PEG had shear moduli between 10-150 kPa. This data demonstrates the ability to increase shear modulus by increasing the weight percent of the monomer mix. By comparison, native articular cartilage has an elastic modulus that ranges from 500-1000 kPa. These data will be used to identify ideal formulations for chondrocyte delivery, with the expectation that targeting the physical properties of the materials to closely match the in vivo environment will provide the best reparative effects.

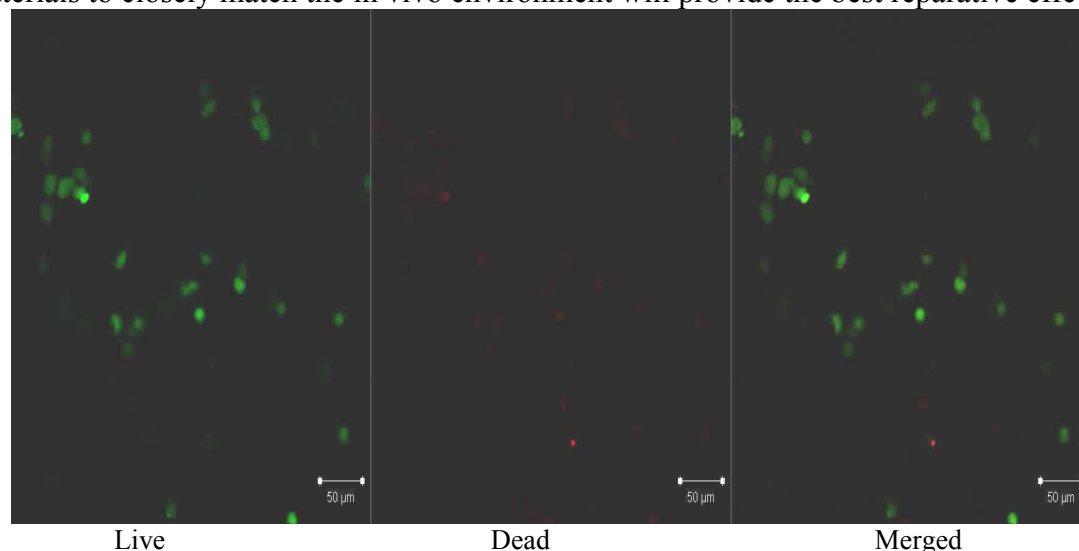


Figure 6. Chondrocytes seeded at 40 million cells/mL continually exhibit around 90% viability from day 0 to day 25 with time points taken every 5 days in 8 arm 10 kDa thiol-ene hydrogels.

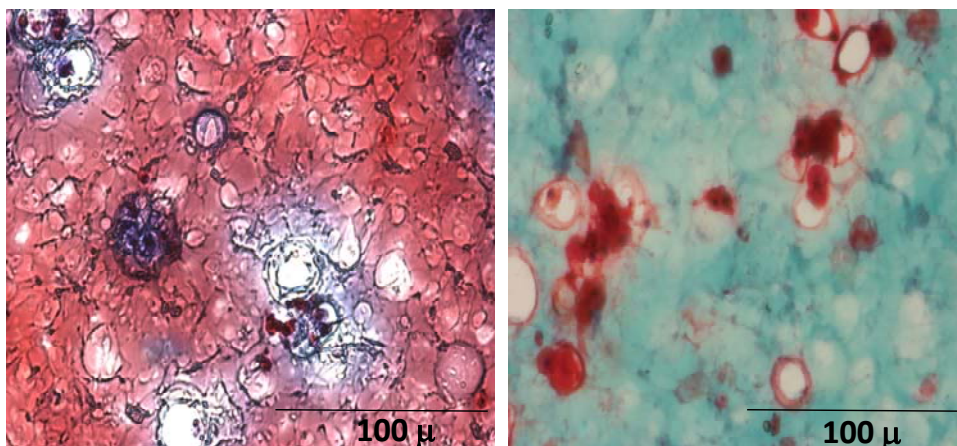


Figure 7. Chondrocytes seeded at 40 million cells/mL show matrix production of glycosaminoglycans and collagen at day 24 in a degradable gel. The matrix production is localized around the cell in this experiment when it is expected to be more diffuse with a better degrading sequence.

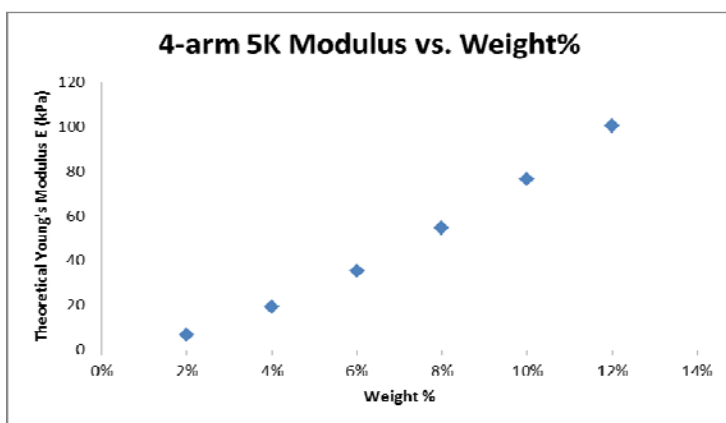


Figure 8a: Increases in weight % of gel lead to increases in theoretical Young's modulus for a 4-arm 5kDA PEG Norbornene gel.

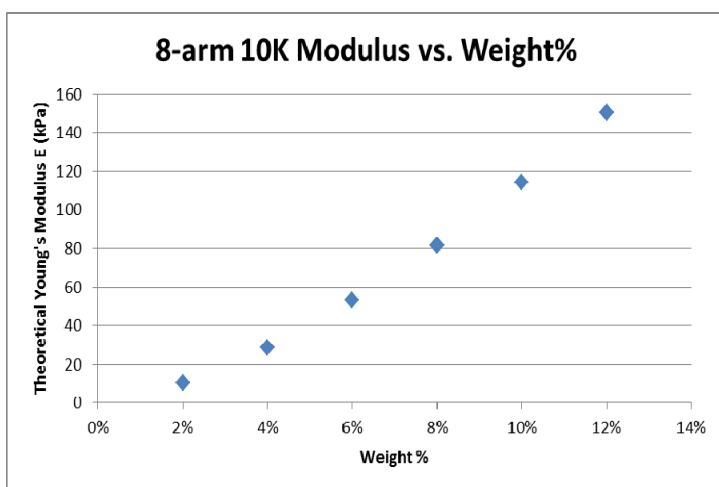


Figure 8b: Increases in wt % of gel lead to increases in theoretical Young's Modulus for a 8-arm 10 kDA PEG Norbornene gel.

Subtask 1.1.c Evaluate cartilage and bone matrix produced in vivo in mice.

- Cells encapsulated in photochemically crosslinked gels implanted in mice for matrix production in vivo.

Results: Swine chondrocytes were encapsulated in 4 percent bovine collagen gels, fibrin gels, and combination fibrin/collagen gels that were placed into rings of devitalized native articular cartilage and photochemically crosslinked with riboflavin and blue light in ambient air. Controls gels were not photochemically crosslinked. New cartilage matrix was formed in vivo in mice after 3, 6, and 12 weeks (Figure 9). New cartilage matrix formation was observed in all gels, both crosslinked and noncrosslinked. Integration of the neocartilage and the devitalized cartilage ring was also noted at the interface (Figure 10). This experiment will be expanded and repeated in year 3. Gels will be made under ambient air conditions and groups will be added under hypoxic conditions.

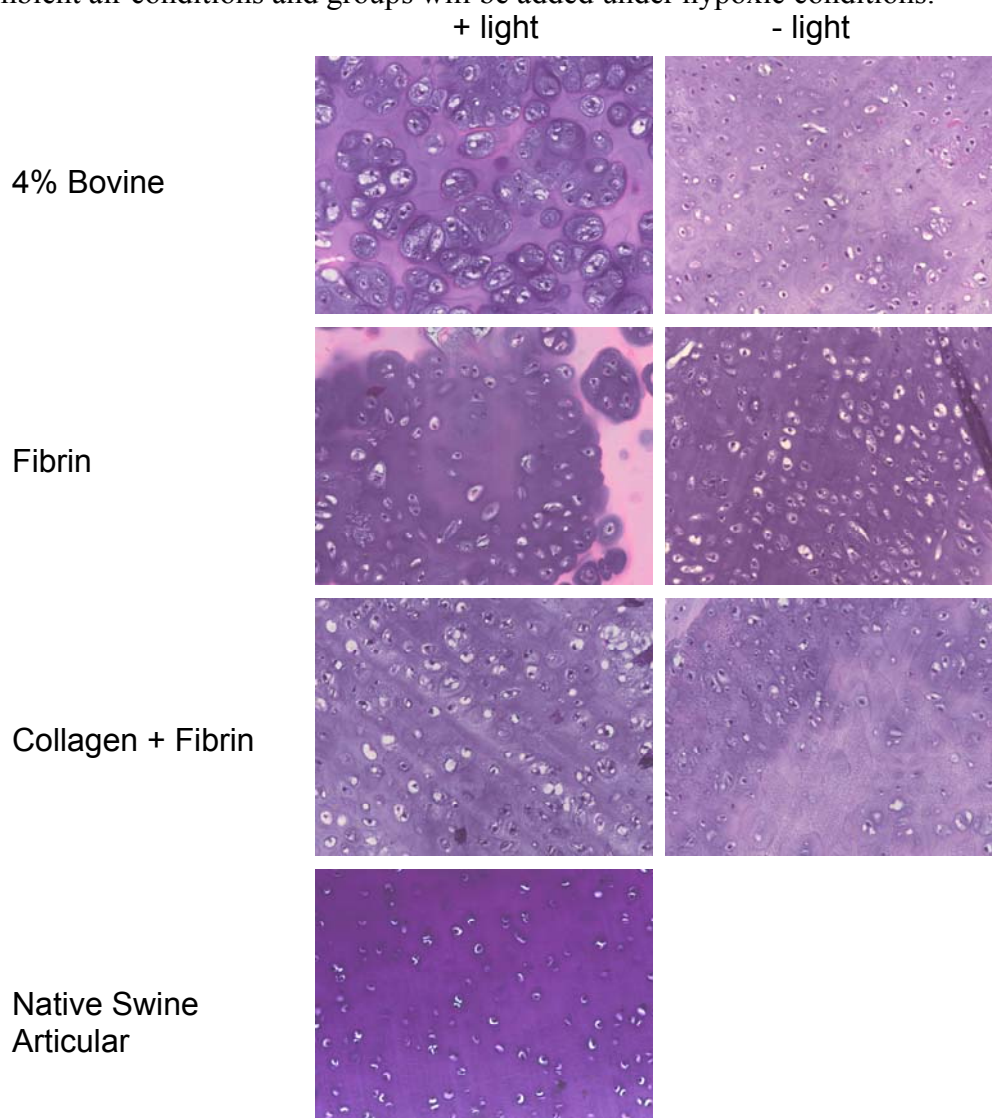


Figure 9. Specimens harvest from mice after 3 weeks in vivo showing the neocartilage that was formed by the cells in each of the gels. The left column are gels that were photochemically crosslinked with light and the right column are the control gels not receiving light. Native swine articular cartilage is shown at the bottom for comparison. (All 200x; H&E)

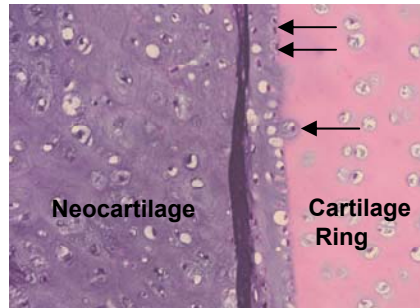


Figure 10. This image shows the interface of the neocartilage and the devitalized cartilage ring. Integration is observed with chondrocytes aligning on the surface of the devitalized cartilage ring and some are migrating into the existing matrix (arrows; 200x; H&E).

Subtask 1.2.a Initiate 3-month pilot study in swine with photochemically crosslinked collagen and PEG gels with bilayer of chondrocytes and osetoblasts

- Cells were encapsulated in photochemically crosslinked gels and implanted in swine to evaluate the survival of the cells in vivo.

Result: We reported on the development of the swine model in year 1 showing that four defects could be made in the patella (trochlear) groove and two defects could be made in the medial condyle. We also showed in a pilot study that matrix was evidenced in the specimen implanted in photochemically crosslinked riboflavin gel at 4 weeks. As reported above, we have made modifications to the gels because the photochemically crosslinked collagen gel was too weak to remain in the defect (see subtask 1.1.a above for data). This has strengthened the gels and will allow for stabilization at the time of implantation into the defects in the large animal model. We have performed a pilot study in 2 swine to evaluate cell survival after implantation of the cell-scaffolds. One pig was evaluated at day 4 and the other at day 14. The appearances of the gels in swine 20385 at day 4 are shown in Figure 11c. The gels were carefully removed from the defects and evaluated with a live dead assay to assess the survivability of the implanted cells. The results are shown in Figure 11d, e, & f for cells in control fibrin gel, photochemically crosslinked fibrin gel, and crosslinked PEG thiol-ene gel. The fibrin control shows large numbers of cells present in the gel with very minimal cell death (red labeled cells) (Figure 11d). The fibrin construct crosslinked with light shows a high overall number of live cells, but more dead cells than the unirradiated control (Figure 11e). The crosslinked PEG gel also shows a high number of live cells with some dead cells interspersed throughout (Figure 11f). This short-term pilot study confirms that the cells survive the gelation and implantation process. Modification described above to perform the photochemical crosslinking in hypoxic conditions may improve cell survival and will be employed in swine in year 3.

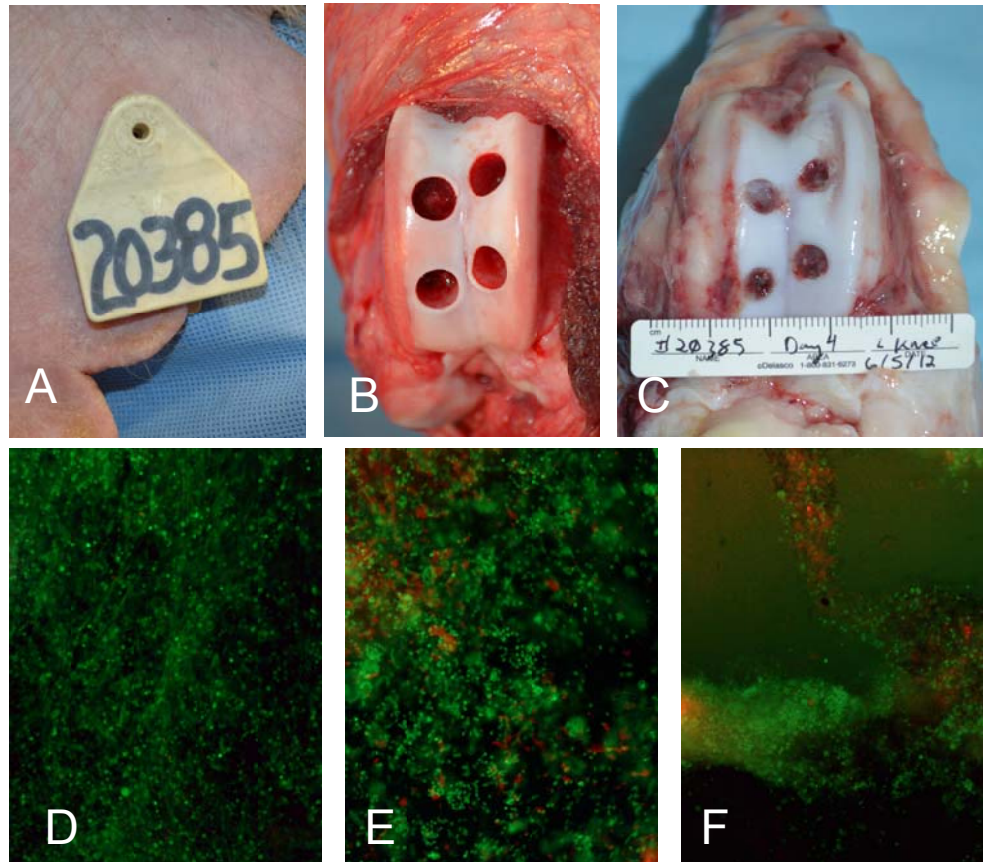


Figure 11. Results from swine 20385 after 4 days in vivo. Four defects (6mmx10mm) are made in the trochlear groove of the knee joint of the swine (b). The defects were filled with noncrosslinked fibrin gel, photochemically crosslinked fibrin gel, and photochemically crosslinked PEG thiol-ene gel (c). Live/dead assays at 4 days show mostly live cells in the noncrosslinked fibrin (d). There is a slight increase in the number of dead cells in the photochemically crosslinked fibrin gel (e) and PEG gel (f).

Task 2 Stimulation of chondrogenesis by stem cells in photochemical gels

Subtask 2.1.a Perform initial study of collagen and PEG gels with stem cells implanted in mice

- Bone marrow MSCs were harvested from donor swine and grown in culture.

Results: BM-MSCs were isolated from swine bone marrow collected by aspiration from the iliac crest of swine. The MSC population was isolated by attachment to plastic culture dishes and propagated in high glucose DMEM. To test the multilineage potential of swine BM-MSCs, the cultured cells were placed into commercial differentiation media from Invitrogen to differentiate them into osteogenic, adipogenic and chondrogenic lineages. The assays for osteogenic and adipogenic assays were conducted on MSCs attached to culture plates. For osteogenic differentiation, the cells were assayed for the production of alkaline phosphatase shown in Figure 12a & b. Cells that were differentiated into an adipogenic lineage were assayed for the presence of lipid in the cells using Oil Red O staining (Figure 12c & d). For chondrogenesis, 250,000 cells were grown in pellet culture and treated with chondrogenic media. The pellets were evaluated histologically to assess the presence of cartilage-like matrix using stains for the presence of charged glycosaminoglycans (Figure 12e, f, & g). The results demonstrate that the isolated swine BM-MSCs were differentiated into the osteogenic lineage demonstrated by the increased production of alkaline phosphatase; into the adipogenic lineage with the formation

intracellular lipid; and the chondrogenic lineage as demonstrated by the production of cartilage-specific extracellular matrix. Plans for year 3 include the combination of cells differentiated to the osteogenic and chondrogenic lineages with scaffolds to promote clinically useful amounts of bone and cartilage for osteochondral repair and regeneration.

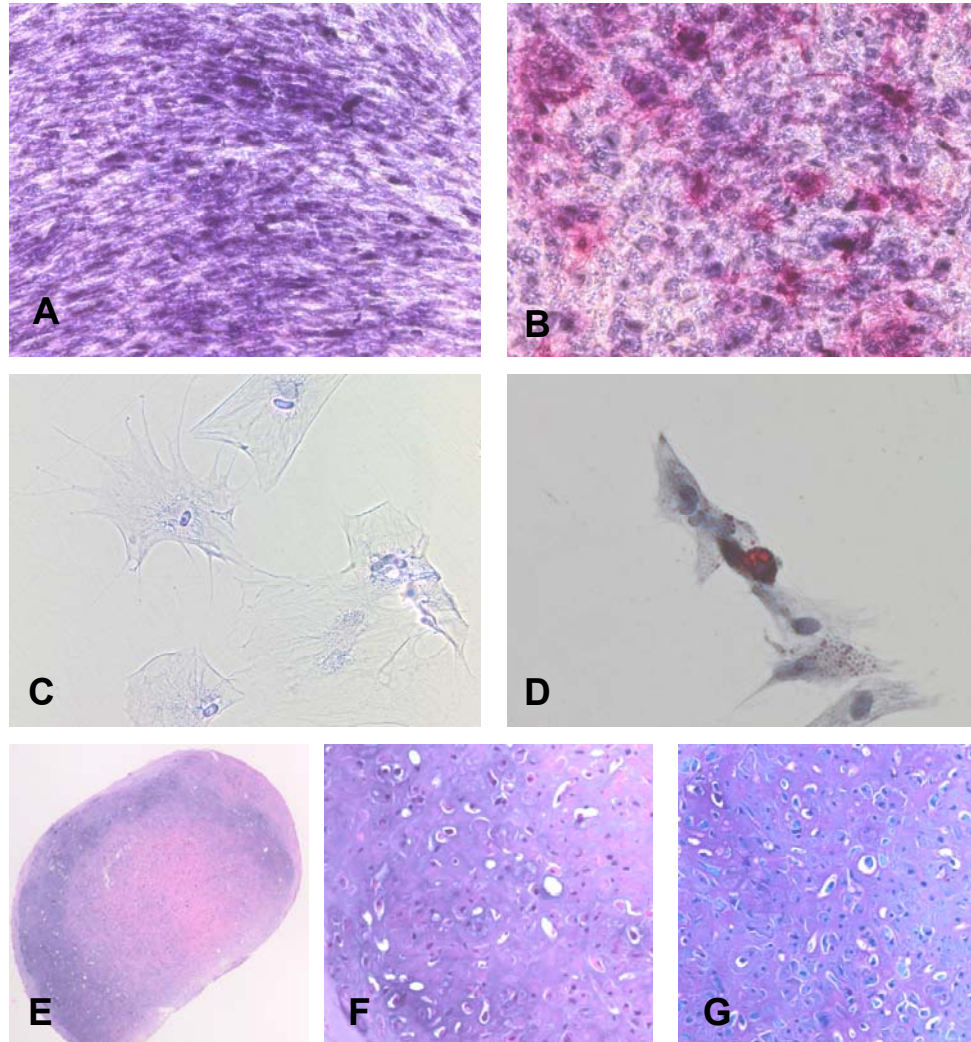


Figure 12. Differentiation of swine BM-MSCs in osteogenic (a&b), adipogenic (c&d), and chondrogenic lineages (e,f,&g). The cells grown in control media without osteogenic factors show no alkaline phosphatase activity (a; 100x), whereas cells treated with osteogenic factors show increased alkaline phosphatase as indicated by the red staining (b; 100x). Cells grown in control media do not have any evidence of intracellular lipid (c; 400x), but cells in the presence of adipogenic media show small red droplets of oil as evidenced with Oil Red O staining (d; 400x). Cell pellets treated with chondrogenic media show the coalescence of cells into a pellet and the presence of cartilage matrix formation (blue staining with H&E; e; 40x). High magnification of the blue-stained areas show chondrocytes encased in lacunae typical of cartilage (f; 400x). Sections from the same tissue block stained with toluidine blue shows GAG rich matrix indicative of cartilage formation (g; 400x).

KEY RESEARCH ACCOMPLISHMENTS

- Modifications of photochemically crosslinked gels:
 - Adding fibrin to the type I collagen solution and photochemically crosslinking the combination gel using Riboflavin as the photoinitiator and blue light (458 nm) increases the bulk modulus of the gels
 - Performing the photochemical crosslinking under hypoxic conditions may improve cell survival
 - Cells are uniformly distributed throughout the crosslinked gels and the cells begin producing pericellular matrix by 2 weeks in vitro in the gels
 - High cell survival and matrix production was noted in the poly(ethylene) glycol thiol-ene gels
- Cell tracking was verified in vivo with DiI labeled cells encapsulated in gels placed in nude mice for up to 8 weeks
- Formation of new cartilage matrix was demonstrated in vivo in mice using photochemically crosslinked gels and swine articular chondrocytes placed into cartilage rings
- Bone marrow-derived mesenchymal stem cells from swine were isolated and differentiated into osteogenic, adipogenic, and chondrogenic lineages with commercial differentiation media containing growth factors inducing differentiation
- Chondrocytes encapsulated in photochemically crosslinked hydrogels can survive the crosslinking and implantation process as shown in swine

REPORTABLE OUTCOMES

Abstract presented (Appendix A):

Photochemical Crosslinking Stabilizes Protein Hydrogels for Cartilage Regeneration.

Omobono MA, Randolph MA, Zhao X, Redmond RW

American Society for Photobiology, Annual Meeting, June 23-27 2012, Montréal, Quebec, Canada

Abstract submitted (Appendix B):

Photochemical Crosslinking Stabilizes Protein Hydrogels for Articular Cartilage Regeneration"

Omobono MA, Jang S, Randolph MA, Zhao X, Redmond RW, Gill TJ

Orthopedic Research Society, San Antonio, February 2013

Manuscript prepared for submission to Biomaterials (C):

Enhancing the stiffness of collagen hydrogels for delivery of encapsulated chondrocytes to articular lesions for cartilage regeneration.

Omobono MA, Zhao X, Furlong MA, Kwon CH, Randolph MA, Gill TJ, Redmond RW,

CONCLUSION

At the conclusion of the second year of this grant award, we have made modifications to the formulations of photochemically crosslinked hydrogels that will be used to deliver chondrocytes to articular cartilage defects in the swine knee joint. Data from year 1 showed that the photochemical crosslinking of collagen gel improved the stability of the gels by making them resistant to collagenase digestion. However, the crosslinking had little effect on the mechanical properties of the gels (i.e., bulk modulus). Since fibrin gels have been used successfully to engineer cartilage by us and other groups, work in year 2 included studies on photochemically crosslinking fibrin hydrogels. We show that fibrin has a bulk modulus two orders of magnitude higher than collagen gel and, when combined with the collagen, the modulus of the combination gel is 20-fold higher than collagen alone. Thus, we may be able to exploit the advantages of both types of gel in a combination gel. Gels containing cells showed the same results, albeit these had lower moduli overall. Another candidate gel is photochemically crosslinked poly(ethylene) glycol gel that is crosslinked with a photoinitiator and ultraviolet light. The crosslinking process of both collagen and PEG permit high cell viability using isolated swine articular chondrocytes. Different mechanical properties can be achieved when using different formulations (e.g. 4-arm vs. 8-arm PEG thiol-ene gels). An initial pilot study of gels implanted into defects validated the potential for cells to survive the photochemical crosslinking process of these gels and implantation into defects in the swine knee. Although we originally planned to implant gels in a 3-month swine study in year 2, the mechanical and biological characteristics of the gels were inferior a year ago. As we described in our testing paradigm shown in figure 1 of this report, gels that do not perform well in vitro or in vivo in mice will be reformulated and tested before moving into large animal swine studies. With the modifications described in this report, we will move forward with large animal testing in year 3.

The goal of task 2 is to isolate bone marrow derived mesenchymal stem cells from swine and encapsulate these cells in the photochemical gels. In year 2 we have isolated swine BM-MSCs and propagated them in culture. To test the multilineage potential of these cells, cultured cells were differentiated into osteogenic, adipogenic, and chondrogenic lineages using commercial differentiation media containing growth factors. The results show that the BM-MSCs can be differentiated into these lineages in vitro. Studies in year 3 will focus on encapsulating BM-MSCs in the photochemically crosslinked gels. Testing will continue with in vitro assessment of matrix specific protein production. Studies will also begin to test these differentiated cells in gels in vivo in mice.

“So What” Section:

In the first and second years of this project our team has developed photocrosslinkable hydrogels that could be used to regenerate cartilage in defects in the articular cartilage surface of joints with the goal of restoring normal joint function. These crosslinkable hydrogels serve as biomimetic polymers that provide a favorable environment for encapsulating chondrocytes (the native cell type found in cartilage) and chondrocyte precursor cells (mesenchymal stem cells). We have successfully formulated collagen and PEG gels, and now a combination collagen+fibrin gel. Whereas we had intended to complete a 3-month study in swine in year 2, the mechanical and biological characteristics were inferior and not suitable for implantation in large animals. According to our iterative testing protocol, work in year 2 focused on improving the mechanical and biological characteristics of these gels. A pilot study in swine has demonstrated proof of principle that this technology permits cell survival during the photochemical crosslinking process and implantation in swine in the short term (i.e., up to 2 weeks). We anticipate that these new modifications will be a useful adjunct for joint surface repair and regeneration.

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APPENDIX A

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“Photochemical Crosslinking Stabilizes Protein Hydrogels for Cartilage Regeneration”

Mark Omobono*¹, Mark Randolph¹, Xing Zhao¹, Robert Redmond²

¹*Plastic Surgery Research Laboratory, Department of Surgery, Massachusetts General Hospital, USA,* ²*Wellman Center for Photomedicine, Harvard Medical School, Massachusetts General Hospital, USA*

Natural proteins are attractive scaffold materials for tissue engineering purposes. Both fibrin and collagen hydrogels have been investigated as scaffolds for encapsulation of chondrocytes to repair articular surface defects by neocartilage generation. Certain criteria must be met for hydrogels to be suitable for this purpose. The hydrogel must support cell viability and chondrogenesis. It should also retain its shape and structure and resist rapid degradation on implantation and it should possess sufficient mechanical stability for practical handling. Spontaneous fibrin and collagen hydrogels lack one or more of these characteristics. We have found that photochemical crosslinking of biological scaffolds such as fibrin and collagen using visible light and photosensitizers, adds some mechanical strength but more importantly, a much greater resistance to enzymatic degradation, allowing the shape and size of the implant to be retained in vitro and vivo. We have also developed a dual crosslinking paradigm whereby a significant (5-10 fold) increase in gel stiffness can be achieved by following the photochemical crosslinking step with non-toxic chemical crosslinking using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide. We report on the use of fibrin and collagen as scaffolds and rose bengal and riboflavin photosensitization using green and blue light, respectively. Encapsulation of chondrocytes can be achieved using all combinations with excellent viability. Mechanical properties, cartilage generation in mouse models and modulation of the process to remove side effects of reactive oxygen species generation will be presented. This mild crosslinking paradigm, which retains cell viability and encourages cartilage formation, has clinical potential for in-situ repair of articular cartilage defects.

APPENDIX B

"Photochemical Crosslinking Stabilizes Protein Hydrogels for Articular Cartilage Regeneration"

Omobono, Mark A.¹; Jang, Seokyoung²; Zhao, Xing¹; Randolph, Mark A.¹; Redmond, Robert W.²; Gill, Thomas J.¹

1. Orthopedic Surgery, Massachusetts General Hospital, Boston, MA, United States. 2. Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA, United States.

CURRENT PRIMARY CATEGORY: Cartilage & Synovium - Tissue Engineering and Repair

KEYWORDS: Articular Cartilage Repair, Cartilage & Matrix Proteins, Chondrocytes, Tissue Engineering, Knee.

INTRODUCTION: Natural proteins are attractive scaffold materials for tissue engineering purposes. Both fibrin and collagen hydrogels have been investigated as scaffolds for encapsulation of chondrocytes to repair articular surface defects by neocartilage generation. Certain criteria must be met for hydrogels to be suitable for this purpose. The hydrogel must support cell viability and chondrogenesis. It should also retain its shape and structure and resist rapid degradation on implantation and it should possess sufficient mechanical stability for practical handling. Spontaneous fibrin and collagen hydrogels lack one or more of these characteristics. We have found that photochemical crosslinking of biological scaffolds such as fibrin and collagen using visible light and riboflavin-5-phosphate (RF5P) photosensitizer adds some mechanical strength as well as, more importantly, a much greater resistance to enzymatic degradation, allowing the shape and size of the implant to be retained *in vitro* and *vivo*. By measuring bulk storage modulus by rheometer, time of degradation using enzymes, and histological staining of devitalized swine cartilage rings loaded with photocrosslinked protein hydrogel in an *in vivo* nude mouse model, we will explore the capacity of this scaffold paradigm for cartilage generation and a potential use in hyaline cartilage tissue engineering. The aims of this study were to determine both the mechanical and biodegradation properties of type-I collagen and fibrin photocrosslinked hydrogels, and to determine whether encapsulated chondrocytes in these gels would generate neocartilage in an *in vivo* nude mouse subcutaneous model using devitalized swine cartilage rings.

MATERIALS AND METHODS: Type-I bovine collagen, fibrin glue, and a 50:50 mixture of collagen and fibrin were combined with 250µM RF5P to create 4.5mm diameter cylindrical hydrogels. Constructs were either non-photocrosslinked (control) or exposed to 20J/cm² of visible blue light. For cell-seeded rheology and *in vivo* experiments, fresh chondrocytes were isolated from the patellar groove and trochlear chondyle of 3-4 month old Yorkshire swine and seeded in hydrogel suspension at a density of 40x10⁶ cells/mL. Cell-free gels were subjected to enzymatic degradation using 0.025%w/v type-II collagenase or 0.001%w/v papain enzyme and measured for the time of degradation, *t*_{deg.} on an incubated rocker. Cell-free and cell-seeded gels were also measured for bulk modulus, *G'*, on a TA Instruments AR-G2 rheometer using a frequency sweep from 1-10 radians/second with constant 2% strain rate and an 1000 µm gap under continuous, room-temperature oscillation. Cell-seeded hydrogels were loaded into devitalized swine hyaline cartilage rings for subcutaneous implantation *in vivo* in a nude mouse for 3 weeks. After harvest these constructs were stained with H&E to explore the morphology of the neocartilage.

RESULTS: Photocrosslinked protein hydrogels showed a trend towards higher t_{deg} values, and composite collagen/fibrin constructs showed a 4-fold increase resistance to collagenase degradation when photocrosslinked (Fig 1). There was no significant difference in G' between photocrosslinked and control groups for single-material constructs, but a 18-fold increase in G' was shown after

photocrosslinking in cell-less composite constructs, as well as a 9-fold increase in cell-seeded constructs (Fig 2). Constructs harvested from nude mice after 3 weeks showed varying degrees of neocartilage growth within the scaffold matrix. Photocrosslinked collagen and photocrosslinked composite constructs (Fig 3A,C) showed little integration into the devitalized ring and stained strongly for proteoglycans in pericellular regions. Control collagen and composite constructs (Fig3B,D) showed contiguous staining and good integration of the neocartilage matrix into the devitalized ring.

DISCUSSION: Photocrosslinking of protein hydrogels increases the mechanical properties of the scaffold, increases its resistance to biodegradation, and permits neocartilage formation *in vivo*.

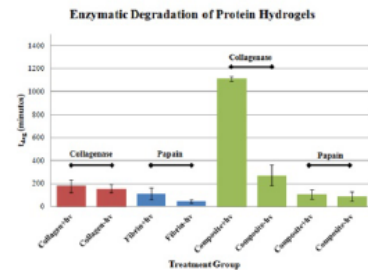


Figure 1: Degradation times of photocrosslinked protein hydrogels of different formulations using type-II collagenase or papain enzyme.

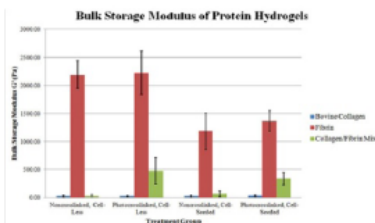


Figure 2: Bulk storage moduli of photocrosslinked protein hydrogels of different formulations using a frequency sweep from 1-10 radians/second with constant 2% strain rate and an 1000 μm gap under

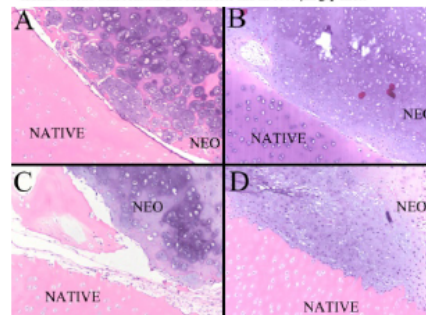


Figure 3: H&E histology of devitalized cartilage rings (NATIVE) loaded with cell-seeded hydrogel scaffold (NEO) after 3 weeks *in vivo*. (A) Photocrosslinked collagen, (B) control collagen, (C) photocrosslinked composite scaffold, (D) control composite scaffold.

APPENDIX C

(for Biomaterials)

Enhancing the stiffness of collagen hydrogels for delivery of encapsulated chondrocytes to articular lesions for cartilage regeneration

Mark A. Omobono^{1,2}, Xing Zhao¹, Michael A. Furlong², Chi-Heon Kwon²,

Mark A. Randolph^{1,3}, Thomas J. Gill³, Robert W. Redmond²

¹Plastic Surgery Research Laboratory, Department of Surgery,, Harvard Medical School,
Massachusetts General Hospital, Boston, MA 01224

²Wellman Center for Photomedicine, Harvard Medical School, Massachusetts General Hospital,
Boston, MA 01224.

³Department of Orthopedics, Harvard Medical School, Massachusetts General Hospital, Boston,
MA 02114

ABSTRACT

Purpose: A photocrosslinked biomimetic hydrogel matrix, used to deliver chondrocytes to an articular defect and support them during extracellular matrix (ECM) generation, offers an improvement over non-matrix-assisted techniques such as autologous chondrocyte implantation (ACI) for cartilage regeneration. Type I collagen is an attractive biocompatible matrix, but the spontaneous gel has very weak mechanical properties. Photocrosslinking of collagen with visible light and a photosensitizer protects the gel from cell-induced shrinkage but does not significantly increase the stiffness of the gel. A stiffer gel would improve its handling characteristics in a clinical setting. This study investigated a dual-crosslinking paradigm, consisting of (a) photocrosslinking with Rose Bengal (RB) and green light, followed by (b) chemical crosslinking with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS), to enhance gel stiffness and reduce gel contraction while retaining chondrocyte viability.

Materials & Methods: Three gel conditions were investigated. In group 1, 50 μ L collagen constructs of 2% (w/v) type I collagen containing 10 μ M RB were allowed to gel spontaneously at 37°C. In Group 2, the spontaneous gels were exposed to green light (532 nm) alone and in Group 3 the photochemical gels were subsequently treated with a 1 hour exposure to 33 mM EDC/6 mM NHS. The above samples and associated controls (n=18) were subjected to 0.25% (w/v) collagenase digestion to evaluate resistance to enzymatic digestion. The storage modulus of each gel was measured using a rheometer. The viability of encapsulated chondrocytes was measured by fluorescent microscopy and live/dead assay in all groups after *in vitro* culture for 10 days.

Results: Chondrocytes were $\geq 95\%$ viable in all constructs at 10 days *in vitro*. Resistance to collagenase digestion increased in the order spontaneous gels (2h) < photochemical gels (3-4 h) < dual crosslinked gels (>24h). The storage modulus of dual-crosslinked constructs was increased 5-fold over that of both photocrosslinked and spontaneous gels.

Conclusions: Photochemical \pm chemical crosslinking of collagen I hydrogel did not reduce encapsulated chondrocyte viability, while the dual-crosslinked collagen gels demonstrated an increase in stiffness and resistance to collagenase. These crosslinked collagen hydrogels could be a useful tool for the practical delivery of encapsulated chondrocytes to articular defects.

INTRODUCTION

Articular cartilage is a complex tissue and presents a significant clinical challenge for natural regeneration. It is populated exclusively by chondrocytes (*1*), but is avascular and lacks the capacity for complete, spontaneous regeneration in response to any focal injury to the contiguous cartilage extra-cellular matrix (ECM). Any procedure aimed at restoring the articular surface is required to (a) create natural hyaline cartilage to fill the defect and (b) allow integration of the neocartilage with existing healthy cartilage.

Among many potential natural and synthetic matrices in tissue engineering, collagen has proven to be useful in hydrogel (2-3), membrane (4-5), and porous scaffold (6-7) form. Collagen is naturally occurring in human tissue, including articular cartilage (8). Collagen molecules self-assemble into a hydrogel matrix at physiological pH and temperature via hydrogen bonding but these spontaneous hydrogels are weak in mechanical integrity and are rapidly digested by enzymatic attack. In an attempt to overcome these drawbacks, we exposed collagen gels containing photo-reactive dyes to visible light to induce photopolymerization of adjacent collagen molecules. These photocrosslinked gels were protected from contraction due to cellular interaction with the matrix (9) and chondrocytes encapsulated in photocrosslinked collagen hydrogels formed hyaline-like cartilage (*10*). However, these gels are relatively soft and difficult to handle, and the aim of this work was to modify the crosslinking process to provide a stiffer collagen hydrogel that would be more user-friendly in ultimate clinical use.

Any increase in gel stiffness must be achieved without affecting the viability of encapsulated chondrocytes nor their ability to remodel the existing type I collagen scaffold and generate neocartilage. The feasibility of implanting a collagen hydrogel in an articular surface defect hinges on the mechanical integrity of the hydrogel during implantation and early repair (up to 4

weeks). During this period chondrocytes must have a scaffold that supports viability and is replaced with newly generated ECM. Photocrosslinked gels may lack the stiffness necessary to withstand extended mechanical loading and an enhancement in construct stiffness could be beneficial.

Chemical crosslinking (11-15) using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) is non-toxic and useful for crosslinking collagen in tissues. EDC, in the presence of NHS, activates carboxyl groups of aspartic and glutamic acid residues of collagen to react with nucleophiles, such as primary amines (lysine and hydroxylysine) and hydroxyl groups, to create zero-length crosslinks (16). Treating collagen hydrogels with EDC/NHS is a potential method of increasing crosslinking and the combination of photo- and chemical crosslinking could yield a hydrogel with improved stiffness. (17).

In this study, we have used dual-crosslinking (photochemical + chemical) of type I collagen hydrogels to test whether the stiffness of collagen gels can be substantially increased to make them useful for articular cartilage defect repair. Effects on mechanical properties of the gel, encapsulated chondrocyte viability *in vitro*, and resistance to collagenase digestion were all evaluated to determine the suitability of this new crosslinking paradigm for articular cartilage regeneration.

MATERIALS and METHODS

Materials. Rat tail type I collagen (11.4 mg/ml) was obtained from BD Biosciences, Bedford, MA. Ham F12, 10% fetal bovine serum. 1% antibiotic/antimycotic liquid and 1% MEM non-essential amino acids were all from Gibco (Carlsbad, CA). Sodium hydroxide (NaOH), Rose Bengal (RB), 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide, N-hydroxysuccinimide and N-

(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) were purchased from Sigma-Aldrich (Natick, MA). Type II collagenase at 245 U/mg was purchased from Worthington (Lakewood, NJ).

Preparation of Collagen Hydrogels. Rat tail type I collagen was mixed in an Eppendorf tube with chondrocyte media [Ham F12 (Gibco, Carlsbad, California) base, 10% fetal bovine serum (Gibco), 1% antibiotic/antimycotic liquid (Gibco), 1% MEM non-essential amino acids (Gibco)], 50 mM stock NaOH (Sigma), and 90 μ M Rose Bengal (Sigma) diluted in chondrocyte media.

The collagen solutions were adjusted to pH 7.2 ± 0.2 using 50 mM NaOH. The final concentrations were 10 μ M Rose Bengal and 2.54 mg collagen / mL.

Fifty μ L of the hydrogel mixture was aliquoted into syringe molds, created by removing the injecting tip from a 1mL Monoject syringe with a razor blade. Spontaneous gelation was induced by incubation at 37°C for 1 hour. Photochemical gels were prepared by exposure to green light from a continuous wave KTP laser (LRS-0532-PFH-000500-05, Laserglow Technologies, Canada, 800mW, 532nm) at 20J/cm² fluence from three different angles to assure equal illumination. Some gels underwent subsequent chemical crosslinking following ejection from the syringe and being submerged in a solution of 33 mM EDC, 6 mM NHS, and 50 mM HEPES for 1 hour. Another group of gels was prepared solely by exposure to EDC/NHS for 60 minutes without prior photochemical crosslinking.

In order to compare concentration and time of exposure to EDC/NHS solution, all collagen gels were prepared in a similar fashion through green laser exposure. Samples were then subjected to increasing dilutions and increasing times of exposure (n=3 per group) according to Table 1.

Collagenase Digestion Assay. Collagen hydrogel constructs were submerged in 10 mL of 0.025% w/v type II collagenase to determine the degree of protection from enzymatic

degradation provided by crosslinking treatments in 15 mL conical tubes (BD Biosciences).

Tubes were placed on a lab rocker at room temperature and checked every 15 minutes to determine the time taken for complete dissolution of the construct. Gels that were still intact after 24 hours were recorded as “undigested”. Three individual trials were conducted with n=6 per trial for a total of n=18 samples per group.

Mechanical testing. The viscoelastic storage modulus, G' , of each collagen construct was measured using a rheometer (TA Instruments AR-G2, 8mm Rough Steel Smart-Swap plate, part #511080.906). Samples were subjected to a frequency sweep from 1-10 radians/second with constant 2% strain rate and an 800 μm gap under continuous, room-temperature oscillation. Shear elastic moduli were calculated from the stress measured, using the equation $G' = (\sigma_0 / \epsilon_0) \cos(\delta)$ where σ_0 is the stress applied, ϵ_0 is the strain measured and δ is the measured lag between phases. Three trials were conducted at n=6 per group per trial for a total of n=18 samples per group.

Chondrocyte viability. Knees were obtained from euthanized, 4-month old Yorkshire swine and dissected under sterile conditions to expose the femoral condyles and the posterior face of the patella. The cartilage was dissected from the knee and digested in 1% w/v type II collagenase solution overnight at 37°C . Solutions were passed through a 100 μm cell strainer and centrifuged at 250g for 10 minutes. The cell pellet was collected and washed twice with fresh chondrocyte media. Cells were counted on a hemocytometer and tested for initial viability using the trypan blue exclusion assay. Viable chondrocytes were plated in monolayer at a density of 2×10^6 cells/150 cm^2 and cultivated at 37°C and 5% CO_2 until 80% confluent. Cultured plates were then exposed to 0.05% trypsin-EDTA (Invitrogen) and cells were collected in chondrocyte media, and washed twice with fresh media.

Cells were then suspended in collagen hydrogel mixture at 1.0×10^7 cells / mL hydrogel (n=4). Gels were molded, incubated, and crosslinked as described above. After crosslinking treatment, gels were submerged in chondrocyte media for *in vitro* culture. After 10 days the gels were submerged in a solution of Live/Dead Viability Assay (Invitrogen), containing $1.6\mu\text{M}$ calcein AM and 200 nM ethidium homodimer-1, for 1 hour. The stained constructs were embedded in O.C.T. Compound (Tissue Tek), frozen at -20°C for 1 hour, and sliced into $10\text{ }\mu\text{m}$ sections using a cryostat (Leica CM3050). These slices were then imaged using a Nikon (model #) fluorescence microscope using FITC (480ex/535em) and TRITC (535ex/610em) filters, and processed with NIH ImageJ software.

Statistics. Results of collagenase and mechanical testing data are reported as mean \pm standard deviation. Significance was calculated using 1-way ANOVA analysis with Bonferroni's Multiple Comparison Test post-test with $p < 0.05$ considered significant. Percentage chondrocyte viability was calculated by counting dead (red) and live (green) cells from the fluorescent photographs. Samples were photographed in triplicate, capturing one central image and two boundary images per sample. Both live and dead cells were counted manually from which the viable fraction of cells was calculated. Manual counting was performed by 5 independent evaluators and results are presented as the mean of the five independent viability percentage for each gel \pm standard deviation.

RESULTS

Resistance to Collagenase Digestion. Full degradation was defined as complete dissolution of the hydrogel by collagenase. Collagen hydrogels photocrosslinked with Rose Bengal and green laser exposure ($t_{\text{deg}} = 3.5 \pm 0.5$ hours) exhibited > 2 -fold increase ($p < 0.0001$) in time of

degradation from the spontaneous gels (1.3 ± 0.3 hours). Dual-crosslinked constructs digested in collagenase solution exhibited no signs of degradation, even after 24 hours. Gels that were crosslinked with EDC/NHS alone for 60 minutes were soft but very resistant to degradation; after 24 hours there were no signs of degradation (Fig. 1). Exposure of constructs to different concentrations of EDC/NHS for different times showed varying degrees of resistance to digestion (Fig 3). Within a treatment dilution group, t_{deg} increased with increasing time of exposure to EDC/NHS. Between treatment dilution groups, t_{deg} increased with increasing concentration of EDC/NHS.

Mechanical Testing. Values for storage modulus in control gels (25.8 ± 1.5 Pa) and storage modulus in photocrosslinked gels (21.4 ± 1.8 Pa) showed no statistically significant difference ($p = 0.0712$). Storage modulus measurements for dual-crosslinked gels were 5-fold higher (117.6 ± 6.9 Pa) than both photocrosslinked and spontaneous gels ($p < 0.0001$). (Fig 2). Gels exposed to dilute concentrations of EDC/NHS (1:10, 1:5) did not show any significant difference in storage modulus from uncrosslinked and photocrosslinked groups (Fig 4). Groups exposed to EDC/NHS diluted 1:2 for at least 15 minutes showed a trend of increasing storage modulus with increasing exposure time.

Chondrocyte Viability. Chondrocytes exhibited $96.1 \pm 2.3\%$ viability in hydrogel implants after 1 week of *in vitro* culture (Fig 5). Cells residing in the peripheral regions of the constructs tended to have a lower viability than those in the central regions of the gels. Overall, the viability far exceeded the target viability (90%) required for encapsulated chondrocytes to produce healthy ECM. (10)

DISCUSSION

Articular cartilage regeneration is a challenging clinical problem. The newest tissue-engineering-based therapy, autologous chondrocyte implantation (ACI), involves biopsy of healthy hyaline cartilage from a non-weight bearing area of the knee to harvest autologous chondrocytes for expansion *in vitro* followed by injection of cultured cells to the cartilage defect. The cells are typically suspended in saline and a periosteal flap is sutured to the cartilage surface. Not only does this repair require multiple surgeries to complete, but there is little data supporting the benefits of ACI versus microfracture (18) or OATS (19). A common problem during this procedure is the leaking of cell-saline suspension from under the periosteal flap out of the target site due to compressive pressure despite sealing the surgical site with suture and fibrin glue. Finding a suitable matrix to support cellular activity and ECM generation is a common problem in tissue engineering (12, 20-23). Native cartilage consists of different types of collagen fibrils, but is almost 50% type II collagen by dry weight (8). Type I collagen is the main structural component in many native tissues in the body, therefore it is an attractive option as a non-toxic, biomimetic matrix to support natural ECM regeneration by chondrocytes. Collagen hydrogels can be formed at 37 °C but these “spontaneous” gels are very soft and are unsuitable for implantation in focal articular cartilage defects.

Photocrosslinking of synthetic polymers typically is well-established and typically uses UV illumination of photoinitiator compounds that react with synthetic monomers to cause chain reaction polymerization via radical reactions. However, for the purpose of cell encapsulation the UV illumination and the materials used would not be appropriate due to inherent cytotoxicity. For this reason we have investigated visible light as the energy source for crosslinking along with non-toxic initiators. Under these milder conditions the initiating event can be electron transfer between excited initiator and monomer and/or energy transfer from photoinitiator to

dissolved oxygen to form singlet oxygen and its subsequent reactions, (e.g. oxidation of histidine and reactions of photooxidized histidine with other amino-containing residues (24)) to form a crosslink (25).

We have previously shown that photochemical crosslinking of a collagen hydrogel provides a scaffold that supports encapsulated chondrocytes and stimulates cartilage-like ECM production (9-10). Photochemical crosslinking certainly stabilizes the matrix and makes it more resistant to enzymatic degradation but provides little in the way of additional mechanical stiffness that would enhance practical handling of the gel and remove the need for a covering material to be affixed over the cartilage defect to prevent loss of unstable gel. Thus, we seek an improved crosslinking mechanism that enhances initial stiffness of these collagen-based hydrogels.

An obvious possibility would be to increase the fluence (photons/cm²) incident on the gel.

However, we have already shown in cell-free collagen gels that there is a plateau in the fluence dependence of crosslinking, suggesting a saturation of all sites that can be photochemically crosslinked, and further exposure does not equate to increased crosslinking. As an aside, this approach would also be complicated by side reactions of the photoinitiator that can generate reactive species that contribute to cell toxicity, as shown in previous studies. Thus, other methods are required and led to the investigation of chemical crosslinking of collagen-based matrices with a combination of EDC and NHS. This method has been shown to increase mechanical stiffness of materials (11-12, 16, 20, 26-27), including organized tissues, without cytotoxic effects.

We investigated this method alone and in combination with photocrosslinking as a method for increasing collagen hydrogel stiffness. Experiments using EDC/NHS crosslinking alone on collagen gels were disappointing. Constructs exposed to this treatment were only loosely

organized and collapsed under their own weight after being ejected from the mold. These gels could not be subjected to storage modulus evaluation. Despite a lack of structural integrity, these gels proved highly resistant to collagenase digestion, showing that crosslinking did in fact occur. . Since the clinical application requires implantation of snug-fitting gels with a defined geometry, crosslinking with EDC/NHS alone is not an option. However, when photocrosslinking was practiced prior to EDC/NHS treatment a stiffer gel construct was obtained that did retain its shape when extruded from the mold. Thus a dual crosslinking paradigm has potential to provide a practical implant for focal defect repair.

Rheometry testing demonstrated that the stiffness (storage modulus) of the gels increased in dual crosslinked gels (117.6 ± 6.9) more than 5-fold from photochemically crosslinked gels (21.4 ± 1.8 Pa)(Figure 2). Previous results, showing the lack of significant difference between the storage modulus of non-crosslinked, spontaneous collagen gels (25.8 ± 1.5 Pa) and photochemically crosslinked gels, were also confirmed as a t-test results in $p = 0.5814$ between these two groups.

We attribute the resulting increase in G' of dual-crosslinked hydrogels to the addition of new chemical crosslinks throughout the collagen matrix with a resulting increase in crosslinking density. The five-fold increase in G' by EDC/NHS on prior photocrosslinked hydrogels, in comparison to the null effect of EDC/NHS on spontaneously-formed collagen gels discussed earlier, is an interesting observation. A possible explanation is that photocrosslinking treatment provides a stabilizing effect to the hydrogel, aligning collagen molecules to make the EDC/NHS crosslinking more efficient. EDC/NHS crosslinking increases stiffness of structured tissues, such as amnion (11), tendon (28), and sheep dermis (29). Therefore, forming a more organized

collagen matrix by photocrosslinking before exposure to EDC/NHS can positively affect the G' of the hydrogel.

Any crosslinking paradigm for ultimate clinical use cannot be toxic to the encapsulated cells. It is clear that chondrocytes are viable after encapsulation in the dual-crosslinked matrix (Figure 5).

Encapsulation of chondrocytes in type I collagen hydrogels, both spontaneous and photocrosslinked, was shown to be non-toxic in previous studies. The dual-crosslinked constructs were shown to be $96 \pm 2\%$ viable, well above the threshold of 90% viability for good ECM generation capacity used in previous studies (10). Dead cells were few in number and confined to the outer extremes of the construct. Cells also appeared to adhere well to the dual-crosslinked matrix.

The gels that underwent dual crosslinking also retarded degradation by collagenase digestion (Figure 1). Protection from proteases is important for matrix stability during early-stage chondrocyte viability and ECM generation, but over time the initial matrix must be remodeled by natural enzymatic activity and deposition of new hyaline cartilage ECM. If not, the crosslinked type I collagen matrix will impede production of neocartilage. We anticipate that there will be an optimum crosslinking level that provides sufficient stiffness to the gel but also allows for gradual enzymatic digestion and remodeling in vivo. We have shown by using different concentrations of EDC/NHS or treatment times that we can fine-tune the stiffness and also the degradation rate.

Although collagenase degradation is affected by exposing the hydrogel to even the most dilute EDC/NHS groups, there is no significant effect on storage modulus until the hydrogel is exposed to higher concentrations. Compared to full crosslinking (60 minutes exposure time and 33 mM EDC / 6 mM NHS) the stiffness of the gel increased at lower concentrations and lower times of

exposure (Figure 3, 4). Further studies are planned to investigate the cartilage generation capacity of encapsulated chondrocytes in gels in these groups.

The increase in mechanical properties after dual-crosslinking treatment was a very positive result. A major concern in articular cartilage engineering is the ability of a construct to withstand biocompressive forces in the knee, which can be up to 3.40 ± 0.18 times patient bodyweight during a normal walking gait (30). Creating a construct that has a higher storage modulus, (stiffer) would be beneficial as long as the increased stiffness does not impede neocartilage deposition within the matrix. Using a construct similar to those tested here may lead to shorter patient immobilization periods, shorter post-operative physical therapy periods, and an overall faster recovery when compared with recovery periods after solution-based cartilage reparative procedures like ACI. The proven viability of encapsulated chondrocytes and the protection against rapid enzymatic degradation that is provided by this dual-crosslinking paradigm may offer a route to a new, matrix-assisted articular cartilage replacement system.

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Table 1: Group designations for EDC/NHS concentration and time of exposure study.

Dilution [EDC]/[NHS] (mM)	15 min exposure	30 min exposure	45 min exposure	60 min exposure
33/6	1-15	1-30	1-45	1-60
16.5/3	2-15	2-30	2-45	2-60
6.6/1.2	5-15	5-30	5-45	5-60
3.3/0.6	10-15	10-30	10-45	10-60

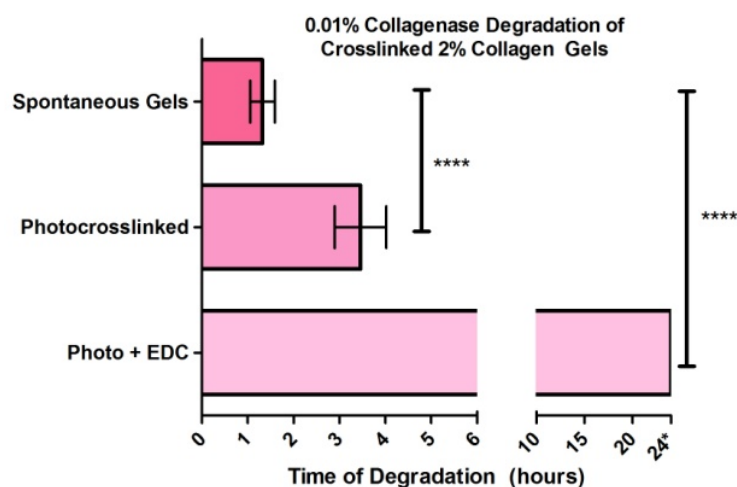


Figure 1: Times of degradation of 2% w/v collagen gels of various crosslinking treatments when exposed to 0.01% w/v collagenase enzyme at room temperature (n=6 per group). Groups are abbreviated using the format of x-y where x is the dilution factor compared to the standard conditions of 33mM EDC and 6 mM NHS and y is the time of exposure to the chemical crosslinker in minutes, e.g. 2-30 refers to a 2-fold dilution and exposure for 30 minutes. Photocrosslinked gels lasted significantly longer (3.46 ± 0.13 h) than spontaneous gels (1.32 ± 0.06 h) ($p < 0.0001$). Gels exposed to dual crosslinking were completely undigested after 24 hours.

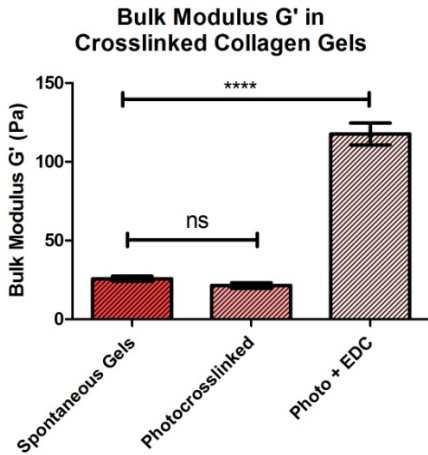


Figure 2: Storage modulus G' as measured by a rheometer of 2% w/v collagen gels of various crosslinking treatments ($n=6$ per group). There was no significant difference between spontaneous gels (25.75 ± 1.53 Pa) and photocrosslinked gels (21.41 ± 1.75 Pa) ($p = 0.58$). There was a significant difference, up to a 5-fold increase in storage modulus when collagen gels were treated with dual crosslinking (117.6 ± 6.93 Pa) ($p < 0.0001$).

Storage Modulus of Collagen Hydrogels with Different EDC/NHS Exposure Concentration, Time

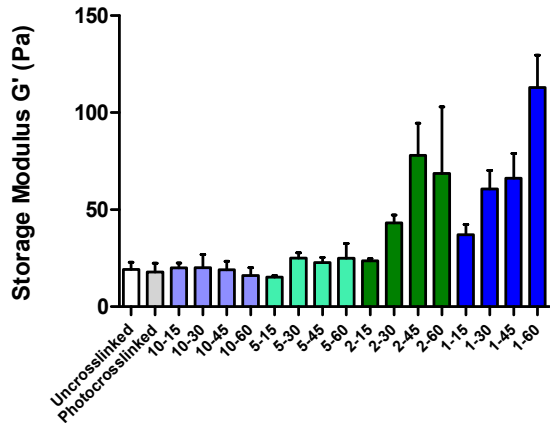


Figure 3: Storage modulus (G') using rheometry of 2% w/v collagen gels treated with different dilutions and exposure times of EDC/NHS. Refer to Table 1 for group designations. There is no statistical significance between gels of groups exposed to lower concentrations of EDC/NHS. The first significant difference is noticed at group 2-30, with varying degrees of stiffness modulation between group 2-30 and maximum crosslinking treatment, group 1-60.

Time of Degradation of Collagen Hydrogels with Different EDC/NHS Exposure Concentration, Time

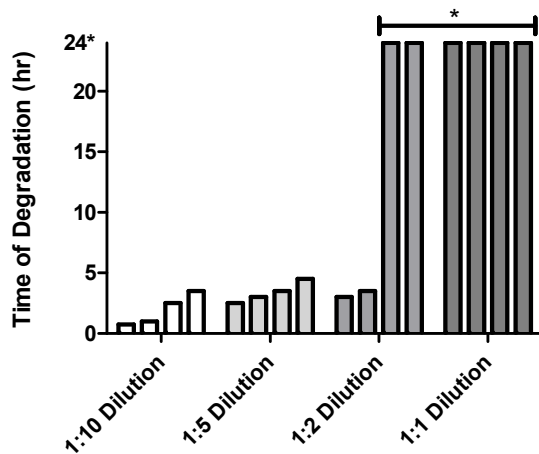


Figure 4: 0.25% w/v collagenase digestion of 2% w/v collagen gels treated with different dilutions and exposure times of EDC/NHS. Refer to Table 1 for group designations. Gels labeled with an asterisk (*) did not digest after 24 hours, when the study was capped.

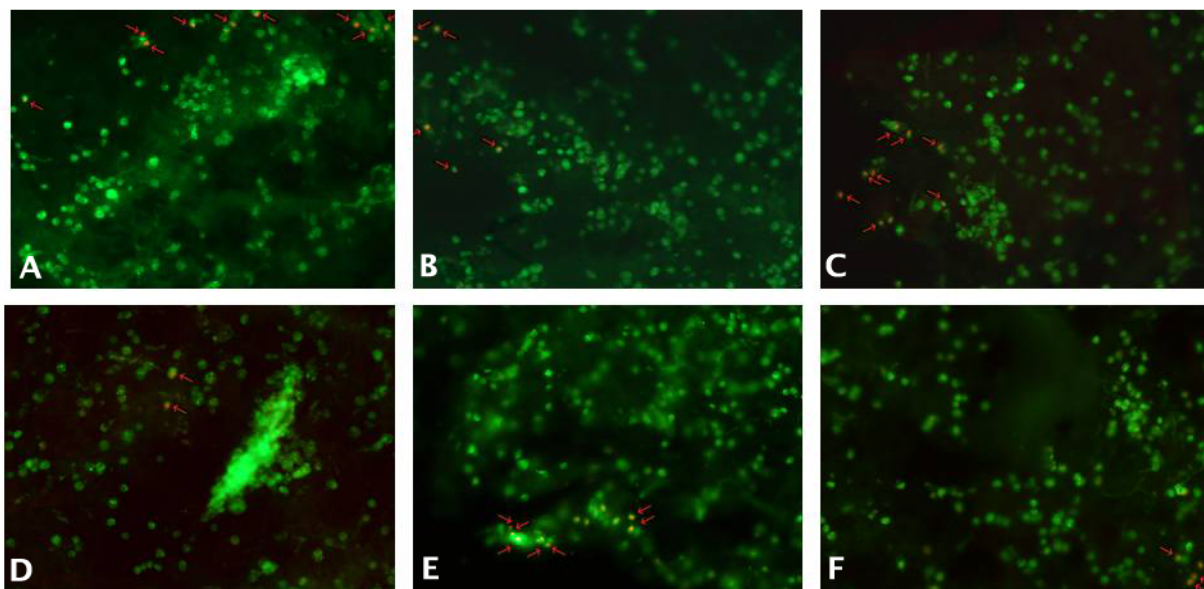


Figure 5: Live/Dead photographs of 6 different constructs after 10 days of *in vitro* culture. Pictures are taken at a height of $750\mu\text{m} \pm 50\mu\text{m}$ from the base of each construct in a $10\mu\text{m}$ cryotome slice. Percentage viability is an average of $96.14 \pm 2.28\%$. Dead cells are denoted by red arrows.